2016

Tumor-Specific Cell Death Induction by Noxa Overexpression for Head and Neck Squamous Cell Carcinoma (HNSCC) Treatment

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TUMOR-SPECIFIC CELL DEATH INDUCTION BY NOXA OVEREXPRESSION FOR HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC) TREATMENT

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology & Biophysics at Virginia Commonwealth University.

By

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Virginia Commonwealth University
Richmond, Virginia
May, 2016

Acknowledgements

I would like to begin by expressing my sincere gratitude to my primary investigator and research advisor *Dr. Hisashi Harada* for all the support and knowledge he has given me. I genuinely appreciate *Dr. Harada's* great mind and love for research. Being a part of *Dr. Harada's* lab provided me with a fascinating research experience and instilled in me a respect for research that will last a lifetime. I would like to thank my laboratory members *Kanika Sharma* and *Thien-Trang Vu* for imparting incredible amount research knowledge. Their uplifting personalities provided an environment that added an enjoyment to everyday of research. I would also like to thank *Dr. Yue Sun* and *Dr. Andrew Yeudall* for the HNSCC cells, and *Dr. Oonagh Loughran* for the NOK. I also am grateful for the discussions provided by *Dr. Sumitra Deb*, *Dr. Swati Deb*, *Dr. Bradford Windle*, *Dr. Steve Grossman*, and members of *Dr. Steven Grant's* laboratory.
I also am thankful for the efforts of my committee members *Dr. Andrei Ivanov* and *Dr. Qinglian Liu.* I also want to thank my friends, colleagues, and professors who have supported me for my project and future goals.

Last but not least, I would like to thank my parents for their continued support and love, which allowed me to pursue a Master's degree in Physiology & Biophysics.

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Abbreviations

HNSCC: Head and neck squamous cell carcinoma

DNA: Deoxyribonucleic acid

RNA: Ribonucleic acid

BCL-2: B-cell lymphoma 2

MCL-1: Myeloid cell leukemia 1

BCL-X_L: B-cell lymphoma-extra large

BAK: BCL-2 homologous antagonist killer

BAX: BCL-2 associated X protein

BAD: BCL-2-associated death promoter

BID: BH3 interacting-domain

CDK2: Cyclin-dependent kinase 2

HPV: Human papillomavirus

shRNA: Short hairpin RNA

cDNA: Complementary DNA

E. coli.: Escherichia coli

TNF: Tumor necrosis factor

TNFR1: Tumor necrosis factor receptor 1

FasL: Fas ligand

FADD: Fas-associated death domain

MOMP: Mitochondrial outer membrane permeabilization

APAF1: Apoptotic protease activating factor 1

BH: BCL-2 homology

DMEM: Dulbecco’s modified eagle medium

FBS: Fetal bovine serum

PI: Propidium iodide

PARP: Poly (ADP-ribose) polymerase

ABSTRACT

TUMOR-SPECIFIC CELL DEATH INDUCTION BY NOXA OVEREXPRESSION FOR HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC) TREATMENT

By: Nicolas Tanner Maxim, B.S.

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Major Director: Dr. Hisashi Harada, Ph. D
VCU School of Dentistry
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The primary focus of this research is the mechanisms of cell death in head and neck squamous cell carcinoma (HNSCC) treatment. These cancers typically originate in squamous cells that line the moist mucosal surfaces of head and neck. HNSCC is commonly treated with a platinum based agent, cisplatin. While the drug does offer strong antitumor effects, its prolonged use often results in tumor-acquired resistance, which limits treatment effectiveness. We have shown that cisplatin treatment induces the expression of a pro-apoptotic BCL-2 family member Noxa, which then initiates caspase-dependent apoptosis through its binding and sequestration of pro-survival protein MCL-1 for its inactivation. Without Noxa induction, cell death is significantly reduced when

treating HNSCCs with cisplatin. The objectives of this study are (1) to determine the molecular mechanisms by which Noxa induces cell death in HNSCC cells; (2) to determine the molecular mechanisms of cisplatin-resistance in isogenic HNSCC cell lines.

We observed an increase of apoptosis by ectopic expression of Noxa in all HNSCC cell lines tested, but not in immortalized human normal oral keratinocytes (NOK), suggesting that Noxa overexpression is sufficient to induce tumor-specific cell death. Noxa-induced cell death was mediated by BAX and BAK activation. BAK activation was mediated through Noxa binding to MCL-1, but not BCL-X_L. Cisplatin-resistant cells induced less Noxa and apoptosis, supporting that Noxa induction is prerequisite for apoptosis induced by cisplatin. Taken together, Noxa induces tumor-specific cell death in HNSCC cells primarily through BAX and BAK activation, which suggests the therapeutic potential of this protein.

INTRODUCTION

1.1 Cancer

Cancer is initiated by a modification of the genes that primarily control how cells proliferate and survive. Cancer is one of the leading causes of death worldwide and its prevalence has increased over the past decade. There were 14 million new cases in 2012, and the estimated number of new cases is 22 million by 2032 [www.cancer.gov]. The most common types of cancer among men and women include lung, head and neck, prostate, colorectal, stomach, liver, breast, and uterine cervix cancer [www.who.org]. With this estimated increase in new cases, it is critical that we continue to make progress in our understanding of the molecular mechanisms of tumorigenesis and cell death.

While genetic alterations cause cancer development, there are many environmental factors that also play a role in the risk of this disease. Such environmental factors include, but are not limited to alcohol, radiation, sunlight, and tobacco. Alcohol consumption is believed to increase ones risk of mouth, throat, esophagus, larynx, liver, and breast cancer [www.cancer.gov]. Ionizing radiation contains wavelengths with enough energy to cause DNA damage, which ultimately leads to cancer [www.cancer.gov]. One prominent radiation threat is the sunlight, which emits UV radiation that over time has the potential to damage skin cells and increases a person's risk of skin cancer. Tobacco use is known to cause a variety of cancers, which include lung, larynx, mouth, esophagus, throat, bladder, kidney, liver, stomach, pancreas, colon and rectum, and cervical cancer [www.cancer.gov]. Tobacco products such as cigarettes and smoke emitted by their usage contain many harmful chemicals that result in DNA damages. While these are some of the primary causes of cancer, there are many other risk factors of

developing cancer such as human immunodeficiency virus (HIV), human papillomaviruses, bacteria, hormones, diet, obesity, and age.

1.2 Head and neck cancer

Head and neck cancer is a phrase used to describe a number of different malignant tumors that arise in the oral cavity, pharynx, larynx, paranasal sinuses, nasal cavity, and salivary glands [2]. Typically these cancers originate in the squamous cells that line mucosal surfaces within head and neck [www.cancer.gov]. For this reason, these cancers are often referred to as head and neck squamous cell carcinomas (HNSCC). HNSCC is the sixth leading cancer worldwide and includes symptoms such as a bump or sore that does not heal, throat irritation, and difficulty of swallowing [www.cancer.gov]. Head and neck cancers make up 3 percent of all documented cancers in the United States [3], and occur twice as much in men as they do in women [1]. HNSCC is diagnosed most often in individuals over the age of 50, many of whom have been exposed to one or more of the risk factors [www.cancer.gov]. The survival rate for individuals diagnosed with head and neck cancer is about 50 percent, and has not changed much in the last 50 years [www.mcancer.org]. It is estimated in year 2016 that 59,340 people will be diagnosed with head and neck cancer and about 12,290 deaths will occur due to the disease [www.cancer.net].

The primary risk factors for head and neck cancer include the use of tobacco and consumption of alcohol, which are estimated about 75 percent of this cancer [4-7]. Studies have shown a direct correlation to the increased use of either one of these carcinogens and an individual's risk of developing cancer within the oral cavity, pharynx, larynx, and esophagus [9]. Another risk factor that has been seen more readily over the past 20 years is human

papillomavirus-16 (HPV-16). This virus often leads to cancers at the back of the tongue and tonsil region, and is spread mainly through oral sex [10].

Before treating individuals with HNSCC, doctors usually perform an assessment to the cancer to determine the place, the stage, its size, and patient's overall health. Knowing the stage of the cancer will greatly aid doctors in choosing the best mode of treatment. To effectively determine the stage of tumor, doctors use the TNM system to place the tumor anywhere from stage zero to stage five. The TNM system considers the tumor size, possible spread of the tumor to lymph nodes, and possible metastasis to other parts of the body [www.cancer.net]. Stage 0 tumor grows only in the location of head and neck where it originates, and has not yet spread to deeper tissue layers or more distant structures. Stage I tumors are estimated to be 2 cm in diameter or smaller, and no other cancer cells are present in surrounding tissues or distant structures. Stage II tumors measure 2-4 cm in diameter, yet no cancer cells are present in surrounding tissues or distant structures. Stage III tumors have grown even larger to 4 cm in diameter or greater. Once reaching stage IV, there are three sub-stages in which the patient may be classified. Stage IVA tumors may be any size and grow into localized structures including lymph nodes on the same side of the primary tumor of head and neck. Stage IVB tumors invade deeper tissues and have spread to lymph nodes but not into distant areas. Stage IVC tumors are any size and have spread to lymph nodes as well as distant areas of the body [www.cancercenter.com].

The main goal of the doctors is to remove or destroy all of the tumors with the least toxicity to the patient. There are several different treatment options such as surgery, radiation therapy, and chemotherapy. For patients with early stage tumor (I or II), surgery or radiotherapy is often used for treatment. In those more progressed patients (stage III or IV), treatment may

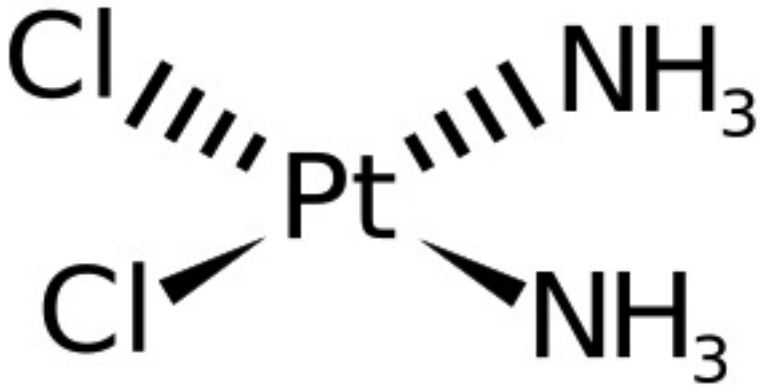
consist of surgery, radiotherapy, and chemotherapy. Types of chemotherapeutic drugs that are used for head and neck cancer include docetaxel, gemcitabine, fluorouracil, carboplatin, and cisplatin (cisplatin being the most commonly used).

1.3 Cisplatin

Cisplatin, a platinum based chemotherapeutic drug with antineoplastic activity, is often used to treat cancers that are metastasized or advanced and unable to be treated by other methods such as surgery or radiation. Cisplatin is widely used to treat cancers including: head and neck, small cell lung cancer, sarcoma, germ cell tumors, lymphoma, and ovarian cancer. Cisplatin (*cis*-$[Pt(NH_3)_2(Cl)_2]$) was created in 1845 by Michele Peyrone, who originally named the compound as Peyrone's chloride. It wasn't until the mid-1900s that Barnett Rosenberg and his colleagues demonstrated cisplatin's ability to inhibit cell division and reduce the size of solid tumors [11,55]. However, even today the complete mechanism as to how cisplatin induces cell death in cancer cells is not fully understood.

Cisplatin is composed of 11 atoms with platinum in the center (Figure 1) [8]. Once intravenously injecting cisplatin, the chemical undergoes aquation to form $[Pt(NH_3)_2Cl(OH_2)]^+$ and $[Pt(NH_3)_2(OH_2)_2)]^{2+}$. The platinum atom of cisplatin covalently binds to the N^7 position of purines to form 1,2- or 1,3-intrastrand crosslinks [14,15]. Cisplatin-DNA adducts result in various cellular responses including replication arrest, transcription inhibition, cell-cycle arrest, DNA repair and apoptosis (Figure 2) [13]. The most common form of cell death seen is through the induction of BCL-2 family-dependent mitochondrial apoptosis and subsequent caspase-3 activation. While the drug does offer strong antitumor effects, it is similar to other chemotherapeutic agents in that its use over a prolonged period of time is limited due to the

toxicity and development of resistance.



http://www.scbt.com/datasheet-200896-cisplatin.html

Figure 1: The structural formula for cisplatin. The chemical name for cisplatin is *cis*-diamminedichloridoplatinum(II)

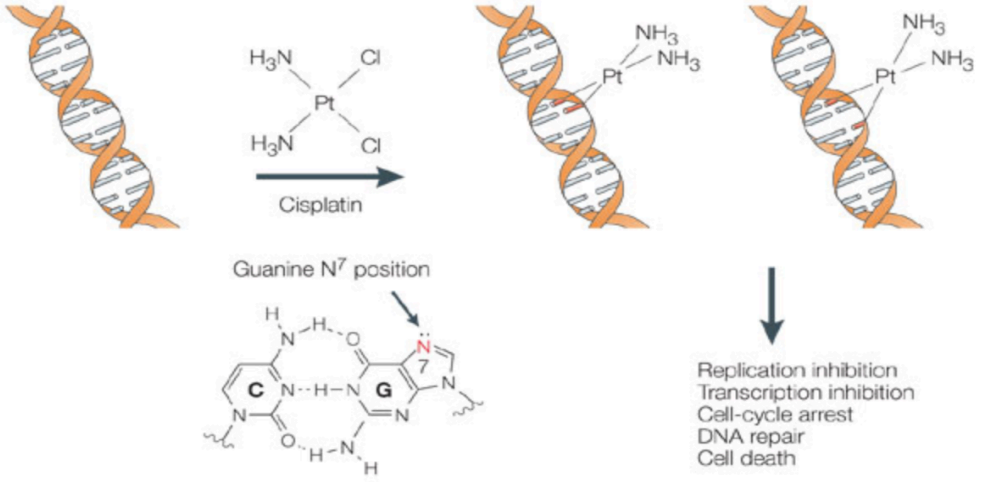


Figure 2: Cellular processing of cisplatin. Formation of cisplatin and its crosslinking with DNA to inhibit replication and to induce cell death [51].

1.4 Cell death

Cell death can be categorized into many categories including apoptosis, autophagy, or necrosis through the analysis of morphological appearances demonstrated by cells as they progress through the pathway. Apoptosis, also known as type I programmed cell death, occurs as a result of caspase activation. With morphological changes, apoptosis is characterized by rounding-up of the cell, retraction of pseudopods, shrinkage, chromatin condensation, nuclear fragmentation, chromosomal DNA fragmentation, and some plasma membrane blebbing. Dying cells proceed through a mechanism that is reversible until a step occurs at which cells have no choice but to die. It is believed that the irreversible step for apoptosis is represented by outer mitochondrial pore formation and permeabilization. Meanwhile, autophagy and necrosis often transpire through caspase-independent pathways. Autophagy is a type of cell death that happens in the absence of chromatin condensation, but in the presence of autophagic vacuolization in the cytoplasm. Necrosis is characterized by a gain in cell volume, swelling of organelles, plasma membrane rupture, and loss of intracellular contents. Necrosis is mostly associated with an unregulated form of cell death that occurs as a result of some pathological condition. However, necroptosis is a term that has been coined to describe a type of regulated necrosis that involves signaling through the kinase RIP1 [16].

Throughout life, the body must maintain homeostasis; one way is though initiating apoptosis to balance out the amount of cell proliferation with cell death. This type of cell death is very important in tissues such as intestinal epithelium and the hematopoietic system, which experience a high turnover rate [17]. Being the most common form of cell death, apoptosis is often the targeted pathway to induce cell death in tumors.

Research suggests that the apoptotic pathway can be divided into two main pathways: the

intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway [17]. The intrinsic pathway can be triggered by various stressful stimuli such as chemotherapeutic drugs that cause the cell to initiate apoptosis. The BCL-2 family proteins (Details are described below) regulate this pathway; they act in a way that alters the outer mitochondrial membrane permeability and allows the release of cytochrome c into the cytosol. Once released, cytochrome c binds to the apoptotic protease-activating-factor 1 (APAF-1) that forms an oligomeric apoptosome. The apoptosome then forms a complex with pro-caspase-9 and cleaves it to release its active form. Activated caspase-9 then acts on other caspase cascades, the most notable of these being caspase-3 activation, which then commits the cell to apoptosis [www.ncbi.nlm.nih.gov]. For the extrinsic pathway, death ligands such as tumor necrosis factor-α (TNF-α), CD95 ligand (Fas ligand), and TNF-related apoptosis-inducing ligand (TRAIL), bind to their respective cell-surface receptors, which results in trimerization of the receptors and recruitment of adapter proteins. This newly formed complex recruits caspase-8, which is activated (cleaved) and then activates effector caspase-3, which commits the cell to die.

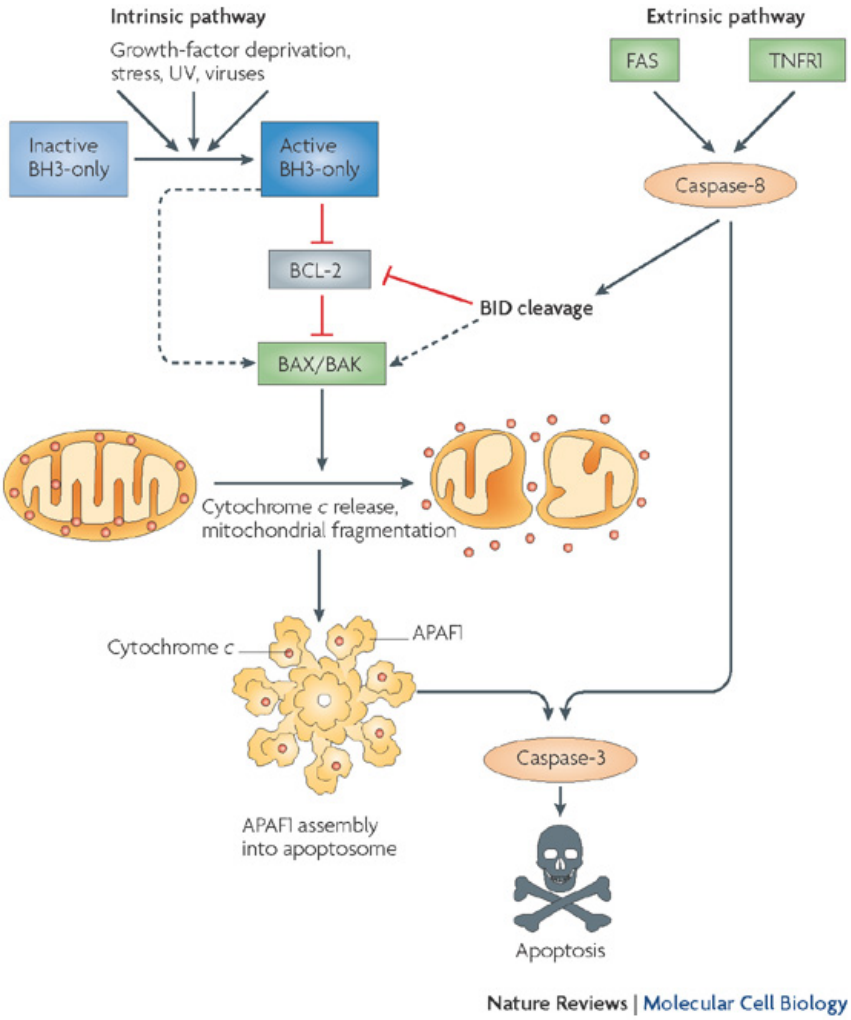


Figure 3: Simplified diagram of the intrinsic and extrinsic apoptotic pathways [50].

1.5 BCL-2 family proteins

The BCL-2 family members play key roles in the regulation of apoptosis through the mitochondrial pathway via protein-protein interactions, which results in inhibition or induction of cell death. These interactions occur between functionally and structurally distinct subgroups at the outer mitochondrial membrane. These subgroups are described as the BH3 (BCL-2 homology 3)-only pro-apoptotic proteins and core BCL-2 family proteins, which contain three or

four BH domains (BH1-BH4). The latter is subdivided into pro-survival and multi-domain pro-apoptotic proteins. These multi-domain pro-survival and pro-apoptotic proteins all share four BH domains. The sequence homology between pro-survival proteins suggests that all of them exhibit a similar fold, where functionally important regions (BH1, BH2, and BH3) are in close spatial proximity [19]. The fold produces a hydrophobic pocket or groove that establishes a crucial interface for interactions with the BH3 domain of pro-apoptotic members of the BCL-2 family.

BAX and BAK make up the multi-domain pro-apoptotic protein group and upon activation (conformational change), they oligomerize and form a pore in the outer mitochondrial membrane. This mitochondrial outer membrane permeability (MOMP) pore allows the release of cytochrome c into cytosol and subsequent caspase-dependent apoptotic events to occur. Multi-domain pro-survival proteins consist of BCL-2, MCL-1, BCL-X_L, BCL-W, and A1 (also known as BFL1 in humans), which act to neutralize or restrain BAX and BAK by protein-protein interactions.

The BH3-only pro-apoptotic protein group consists of Noxa, BAD, BID, BIM, and several others, which are induced transcriptionally or post-translationally upon the exposure to cytotoxic stresses that exceed the apoptotic threshold. These BH3-only proteins act in two ways: to neutralize pro-survival proteins or directly activate BAX and/or BAK. To neutralize pro-survival proteins, the BH3-only protein's BH3 amphipathic helix binds to the hydrophobic pocket of pro-survival proteins (BCL-2, BCL-X_L, MCL-1), which prevents these pro-survival proteins from interfering with BAX and BAK oligomerization for pore formation. BID and BIM are the BH3-only proteins that seem to have the unique ability of directly activating BAX and BAK to induce apoptosis. Due to subtle differences in BH3-only protein's BH3 domains and the grooves to which they bind, some BH3-only proteins such as BAD (BCL-2 antagonist of cell

death) and Noxa are selective for the binding to specific pro-survival proteins. For example, Noxa has a much stronger affinity for MCL-1 (myeloid cell leukemia sequence 1) over any of the other multi-domain pro-survival proteins such as BCL-2 or BCL-X_L.

BAX and BAK proteins act in a similar manner to the BH3-only proteins in that they can bind to the hydrophobic pocket of multi-domain pro-survival proteins with their BH3 domain [49]. However, upon increased expression levels of BH3-only proteins, competition increases between the BH3-only proteins and the pro-survival proteins, which causes an increased rate of apoptosis.

In healthy cells, BAK is located at the mitochondria and is restrained by MCL-1 or BCL-X_L. BAX is located predominantly in the cytosol and restrained by a variety of the pro-survival proteins, but upon activation by cytotoxic stimuli, it gets relocated to the outer mitochondrial membrane. The detailed mechanism as to how this process occurs is not yet understood.

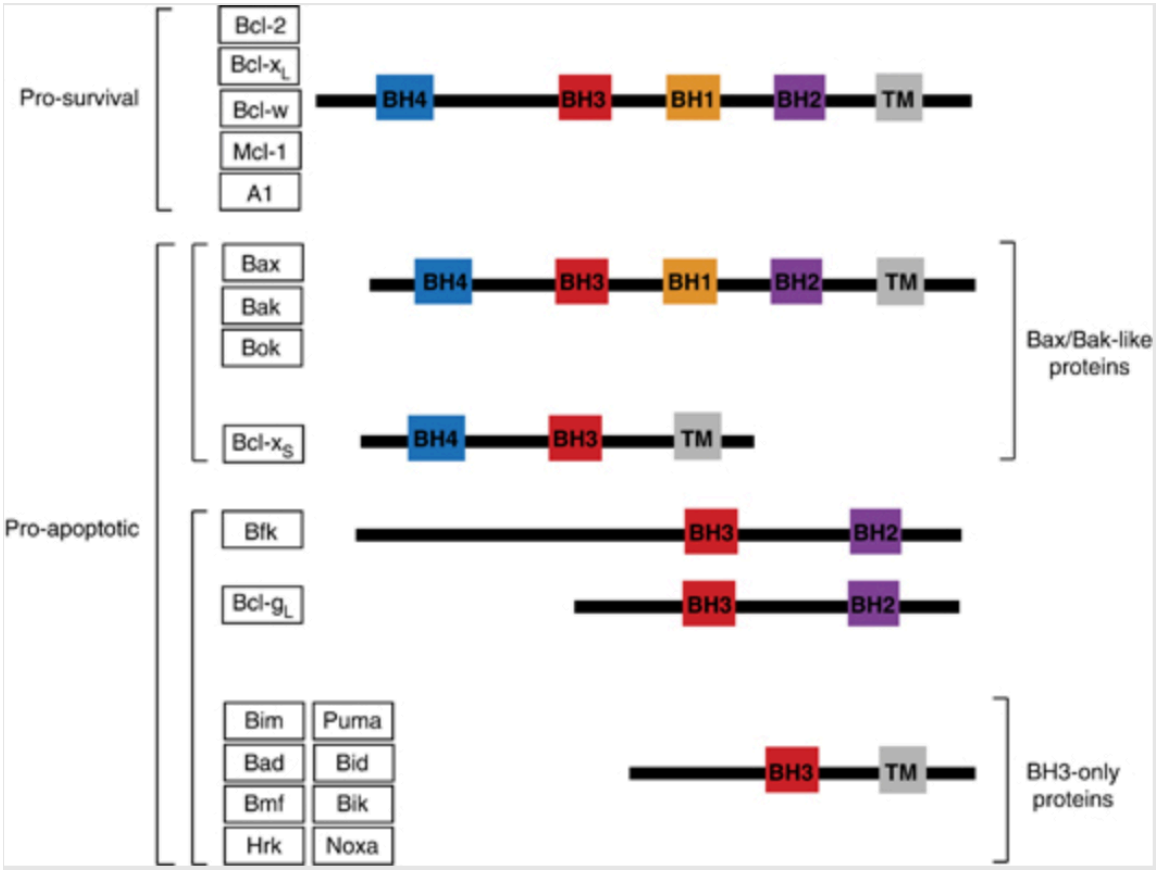


Figure 4: A list of the BCL-2 family proteins. Proteins are separated into pro-survival and pro-apoptotic groups. The major differences between the proteins are the number of BH domains that they contain [20].

1.6 MCL-1 and Noxa

MCL-1 was discovered in 1993 in differentiating myeloid cells [21]. Since then, MCL-1 has been shown to play a key role in the regulation of apoptosis and cell survival in immortalized and tumor cells. The protein is composed of 350 amino-acid residues in human and shares several BH domains with other BCL-2 family members. While BCL-2 and BCL-X_L contain BH domains 1-4, MCL-1 lacks BH4 domain. The structure of MCL-1 protein contains a C-terminal transmembrane domain that functions to localize MCL-1 to various intracellular membranes,

with the most common being the outer mitochondrial membrane [22]. The N-terminus of MCL-1 is comprised of two PEST domains, which are quite prevalent in rapidly turned over proteins. This rapid turnover rate can be greatly attributed to the ubiquitin-proteasome pathway. While MCL-1 expression levels vary among cells, the more differentiated apical layers of epithelia such as prostate, breast, colon, and lung epithelia, tend to have the highest levels [23]. Expression levels of MCL-1 are determined by various cytokines and growth factors, which signal to the cell to survive and differentiate [24]. Many pathways have been shown to induce transcription of MCL-1 through the specific transcription factor response elements in the promoter region of MCL-1, such as mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 (PI3K), and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) [24,25]. Furthermore, direct phosphorylation sites along the PEST domain also control the expression levels of MCL-1. During apoptosis MCL-1 is often down-regulated, which could be a result of caspase cleavage. Activated effector caspases have the ability to cleave MCL-1 at the N-terminal end within the PEST domain. Upon cleavage, MCL-1 pro-survival function is lost, and it can no longer sequester BAK to prevent apoptosis. Overall, MCL-1 is a highly regulated protein and its expression levels are often in direct correlation with cell death and survival.

Noxa, which was identified in 2000 as a p53 target gene, is composed of 54 amino-acid residues in human. The protein is a pro-apoptotic BH3-only protein that also contains a mitochondrial targeting domain at the C-terminus end. Under cytotoxic stress, Noxa has been shown to localize to the outer mitochondrial membrane, where its BH3 domain sequesters and binds to pro-survival BCL-2 family member MCL-1. This recruitment of MCL-1 initiates its phosphorylation at Ser64/Thr70 sites and subsequent ubiquitination, which results in proteasome-mediated degradation [28,33]. This Noxa-mediated phosphorylation of MCL-1 occurs

at the mitochondria and is predominantly regulated by CDK2 [33]. Furthermore, the Noxa/MCL-1 interaction is sufficient to inhibit the function of MCL-1, and causes the release of BAK and subsequent activation (conformational change). Upon activation, BAK is able to oligomerize and form a pore in the outer mitochondrial membrane. Cytochrome c is then released into the cytosol, which ultimately results in caspase-9 activation. To confirm that Noxa plays a critical role in the induction caspase-dependent apoptosis, endogenous Noxa induction was blocked, which correlated with a decrease in the levels of apoptosis. It has been demonstrated that Noxa is capable of being induced by p53-dependent or -independent apoptosis [26]. For example, the use of a DNA damaging agent cisplatin results in p53-dependent Noxa up-regulation, but under hypoxic conditions HIF1α is capable of inducing Noxa independent of p53 [29,31].

1.7 Clinical problem

Over the past three decades, there have been minor advancements with the treatment success of patients diagnosed with head and neck cancer. While treatment options are becoming more personalized from patient to patient, more often than not the three types of standard treatment are used (surgery, radiation, chemotherapy). If the tumor is diagnosed as a stage II-IV, a chemotherapeutic drug such as cisplatin is commonly used to shrink the tumor before radiation or surgery is performed. While cisplatin usage normally results in initial reduction in tumor size, one of the major problems regarding the use of cisplatin is the cancer's ability to develop a clinical drug resistance. This cellular drug resistance may occur as a result of any of the following: 1) decreased uptake of cisplatin, 2) increase in metallothioneins (MT), 3) increase in glutathione and/or glutathione-S-transferase, 4) increased DNA repair, or 5) increased tolerance to unrepaired DNA lesions [34]. The factors listed have a plethora of mechanisms associated with,

which makes it difficult to pinpoint exactly where the cisplatin-resistant phenotype of cancer cells derives from. Furthermore, the mutations that make these cells resistant may vary between cell types and patients.

To overcome the acquired resistance, researchers are currently focusing on the discovery of potential molecular targets for cancer therapy. One example of a molecular target is the epidermal growth factor receptor (EGFR). EGFR is a well-documented proto-oncogene that promotes tumor progression and its expression levels are elevated in >95% of HNSCCs [35]. With this knowledge, EGFR seems to be a good target for patients with acquired cisplatin resistance. EGFR-targeted therapies include the use of monoclonal antibodies (mAb) and tyrosine kinase inhibitors (TKIs). Monoclonal antibodies serve to block the extracellular ligand-binding domain and the TKIs inhibit the activation of the cytoplasmic tyrosine kinase of EGFR [35]. However, unless paired with a high-dose of radiation, the EGFR-specific mAb (cetuximab) or TKIs alone provides only a limited efficacy [36,37]. Therefore, researchers have continued to search for other molecular targets to overcome acquired resistance to chemotherapeutic drugs such as cisplatin.

1.8 BCL-2 inhibitor

After studying the genetic background of various drug resistances in hematopoietic malignancies and solid tumors, the overexpression of pro-survival BCL-2 family proteins are often implicated [39-41]. For this reason, research has quickly started focusing on developing small molecule inhibitors exclusive for pro-survival BCL-2 proteins. By using these small molecule inhibitors in combination with cisplatin, doctors may be able to lower the toxicity of the treatment by reducing the amount of cisplatin used [38]. These BCL-2 protein inhibitors are developed to mimic the BH3 domain of BH3-only pro-apoptotic proteins. The small molecule

inhibitors are designed to compete with the already present BH3-only proteins for binding to pro-survival BCL-2 proteins, which cause a release and activation of BAX and BAK. These BCL-2 inhibitors do not directly trigger cytochrome c release from the mitochondria, but rather decrease the cellular apoptotic threshold, which makes them useful when in combination with standard therapies.

One of these small molecule inhibitors is ABT-737 and orally bioactive ABT-263, which have sub-nanomolar affinities for BCL-2 and BCL-X_L but not for MCL-1. Like other BCL-2 inhibitors, ABT-263 requires BAX and BAK function within the targeted cancer for the drug to be effective [38]. Research has shown that ABT-263 is specific to tumor cells and therefore serves to keep low cytotoxic levels within the patient. However, the main issue with ABT-263 is that it does not have a strong affinity for MCL-1, which is known to be a key anti-apoptotic protein in tumor cells. Future research may lead to the development of a small molecule inhibitor that has strong affinity for MCL-1. Combination therapy with the use of ABT-263 and another MCL-1 inhibitor could prove successful in the treatment of HNSCCs.

HYPOTHESIS

Cisplatin induces antitumor effects through several mechanisms, although the most notable mechanism involves the generation of DNA lesions that result in induction of BCL-2 family-dependent mitochondrial apoptosis. One of these BCL-2 family pro-death proteins that are induced is Noxa, which binds to pro-survival BCL-2 family protein MCL-1, to inactivate its function and release pro-death protein BAK to induce apoptosis. **We hypothesize that Noxa overexpression alone is capable of inducing cell death for HNSCC treatment.**

SPECIFIC AIMS

3.1 Determine the molecular mechanisms by which Noxa induces cell death in HNSCC cells.

Cisplatin toxicity levels on eight HNSCC cell lines will be measured. Lentivirus-mediated shRNA (knockdown of Noxa) will be introduced to examine the involvement of Noxa in cisplatin-induced cell death. Noxa will be overexpressed in eight HNSCC cell lines by lentivirus-mediated transfection. Knockdowns of pro-apoptotic BAX and/or BAK will be conducted with lentivirus-mediated shRNA to determine their function in Noxa-mediated cell death. Protein interaction among BCL-2 family members will be examined by immunoprecipitations followed by Western blot analysis. Cell death will be determined by Western blot analyses or Annexin V-propidium iodide (PI) staining followed by fluorescence-activated cell sorting (FACS) analyses.

3.2 Determine the molecular mechanism of cisplatin-resistant isogenic HNSCC cell lines.

Cells will be treated with cisplatin over a prolonged period of time to mimic tumor-acquired resistance seen in patients. Cells will be infected with lentivirus harboring Flag-tagged Noxa cDNA to overexpress Noxa and see if cell death sensitivity is recovered. Cell death will be analyzed using Western blot analyses and Annexin V-propidium iodide (PI) staining followed by FACS analyses.

MATERIALS AND METHODS

4.1 Cell lines and cell culture

HN4, HN12, HN22, HN8, HN30, and HN31 head and neck squamous cell carcinoma (HNSCC) cells were provided by Dr. Andrew Yeudall (Augusta University, GA), UMSCC1 and UMSCC6 cells were provided by Dr. Yue Sun (Virginia Commonwealth University), and normal oral keratinocytes (NOK) were provided by Dr. Oonagh Loughran (Virginia Commonwealth University). All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies) with addition of 10% heat-inactivated fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA) and 5% 100 μg/mL penicillin G/streptomycin (Invitrogen) at 37°C in a humidified, 5% CO_2 incubator. 293T cells were purchased from the American Type Culture Collection (Manassas, VA). HN30/sh-Noxa, HN31/sh-Noxa, HN31 shC, HN31 shBAX, and HN31 shBAK cells were maintained with 2 μg/mL of puromycin (InvivoGen, San Diego, CA) for selection.

4.2 Plasmid transfection and lentivirus infection

The lentiviral short-hairpin RNA (shRNA) expressing constructs were purchased from Open Biosystems (Huntsville, AL) or Sigma-Aldrich (St. Louis, MO). Flag-tagged Noxa cDNA was cloned into pCDH-EF1-MCS-IRES-neo (System Biosciences, Mountain View, CA). The constructs were transfected into 293T packaging cells along with the packaging plasmid (Addgene; Cambridge, MA) and lentivirus-containing supernatants were used to infect HNSCC cell lines.

4.3 Chemicals and antibodies

Cisplatin (sc-200896) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for BIM, BCL-X_L (54H6), Cleaved PARP (Asp214), GAPDH (D16H11), Cleaved Caspase -3 (Asp175), HRP-linked anti-mouse IgG, and HRP-linked anti-rabbit IgG were from Cell Signaling Technology (Beverly, MA); MCL-1 (ADI-AAP-240-F) from Enzo Life Sciences (Farmingdale, NY); Alpha-Tubulin (sc-8035), BAX (sc-493), and p53 (sc-126) from Santa Cruz Biotechnology (Santa Cruz, CA); Monoclonal BAK (AB-1) and BAK (06-536) from Millipore (Darmstadt, Germany); Noxa (114C307.1) from Thermo Fisher Scientific (Waltham, MA); Monoclonal Anti-BAX (6A7) and BCL-2 (B3170) from Sigma-Aldrich (Saint Louis, Missouri). ECL 2 Western blotting substrate (80196) was purchased from Thermo Scientific (Rockford, IL). Cell proliferation reagent WST-1 was purchased from Roche Diagnostics (Mannheim, Germany).

4.4 Western blot and immunoprecipitation analysis

To perform Western blots, whole HNSCC cell lysates were prepared with CHAPS lysis buffer [20 mM Tris (pH 7.4), 137 mM NaCl, 1 mM dithiothreitol (DTT), 1% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate), 1:200 ratio of protease inhibitor cocktail (Sigma Aldrich), and 1:100 ratio of phosphatase inhibitor cocktails 2 and 3 (Sigma)]. Protein concentrations from the lysates were measured by spectrophotometric analysis with the Bradford method (Bio-rad, Hercules, CA), and equal amounts of samples were loaded into SDS-polyacrylamide gels. The gel was electrophoresed at 180 volts for approximately 45 minutes and the proteins were transferred onto a nitrocellulose membrane (Fischer Scientific, Pittsburgh, PA) with 100 volt for one hour. The nitrocellulose membrane was then blocked with a blotting

solution [5% skim milk in PBST (1 x PBS with 0.1% Tween-20)] for 20 minutes, and specific primary antibodies were incubated with the membrane for overnight at 4°C. The incubated membrane was washed with PBST for at least five minutes for three times and then either HRP-linked anti-mouse IgG or anti-rabbit IgG antibodies were incubated with the membranes for one hour at room temperature. The membranes were washed three times with PBST for five minutes. The membrane was then developed using Pierce ECL- 2 Western Blotting Substrate (Thermo Fisher Scientific).

To perform immunoprecipitation (IP), 500 μg of protein sample was prepared to match a total volume to be 500 μL. The samples were then incubated with the appropriate antibodies for overnight on a rotating table at 4°C. After the incubation, 25 μL of agarose beads with Protein A/G UltraLink Resin (Thermo Fisher Scientific) were added and incubated for 1 hour at 4°C. Supernatants were then removed by centrifugation for 30 seconds at 6000 rpm and 1 mL of CHAPS buffer was added and incubated again on the rotating table for 5 minutes at 4°C. This step was repeated three times. All of the supernatant was aspirated and then the beads were re-suspended with 15 μL of 5x sample buffer. The samples were loaded to SDS-PAGE gels.

4.5 Cell toxicity (WST-1) assay

Head and neck squamous cell carcinoma cells were seeded in triplicate in microtiter plates (96 wells) with $1x10^4$ cells per well in 150 μL medium. The following day, cells were treated with different concentration of cisplatin and 48 hours later, 2 μL of WST-1 reagent were added to the cells. The WST-1 assay is based on tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3- benzene disulphonate). It is used to determine the cytotoxicity of cisplatin by measuring the absorbance of the samples with a microplate enzyme-

linked immunosorbent assay (ELISA) reader (Promega Life Sciences).

4.6 IC_{50}

IC_{50} (half maximal inhibitory concentration) is a term that represents an amount of specific substance (inhibitor) needed to inhibit 50 percent of cell's biological or biochemical mechanisms, i.e. an enzyme, receptors or microorganism. IC_{50} for cisplatin cytotoxicity was calculated for each HNSCC cell line by obtaining quantitative measurements from the microplate ELISA 450nm reader. The measurements were entered in Excel sheets to calculate the IC_{50}.

RESULTS

5.1 HNSCC cell lines and their cisplatin sensitivity, IC_{50}

Table 1 contains a list of head and neck squamous cell carcinoma cell lines that were used in this study. The IC_{50} of the cell lines with cisplatin treatment was analyzed with WST-1 assay. WST-1 (tetrazolium salt) is cleaved to form soluble formazan when cells are metabolically active, which can then be quantitated with a scanning multi-well spectrophotometer at 450nm (Figure 5).

It was found that p53 mutated cell HN31, and p53 wild-type cells, HN30 and UMSCC1 possessed the lowest IC_{50}. p53 truncated cells, HN4 and HN12 had the highest IC_{50}, and p53 deleted cells, HN22, HN8 and UMSCC6 had an IC_{50} between p53 truncated and p53 wild-type cells (Table 1 (a-c [53]) and Figure 6 (a-f [53])).

TABLE 1. HNSCC Cell Lines				
Patient	**Cell Line**	**Origin**	**p53 Status**	**IC_{50} (μM) Cisplatin**
A	**HN4**	Base of Tongue	Truncated (Non-functional)	75
	HN12	Lymph node	Truncated (Non-functional)	63
B	**HN22**	Epiglottis	Deleted	50
	HN8	Lymph node	Deleted	50
C	**HN30**	Pharynx	Wild-type	21
	HN31	Lymph node	Mutated	20
D	**UMSCC1**	Floor of mouth	Wild-type	15
E	**UMSCC6**	Base of tongue	Deleted	40

Table 1: Cell lines harvested from the lymph node are post-metastatic. The p53 genes in HN4 and HN12 cells contain a mutation at the splicing donor site of exon 7 [32], thus p53 is truncated and non-functional. HN31 contains a mutation within the p53 gene, but the gene retains some function [54]. The p53 genes in HN22, HN8, and UMSCC6 cells are deleted, but HN30 and UMSCC1 cells have wild-type p53 genes [28,33].

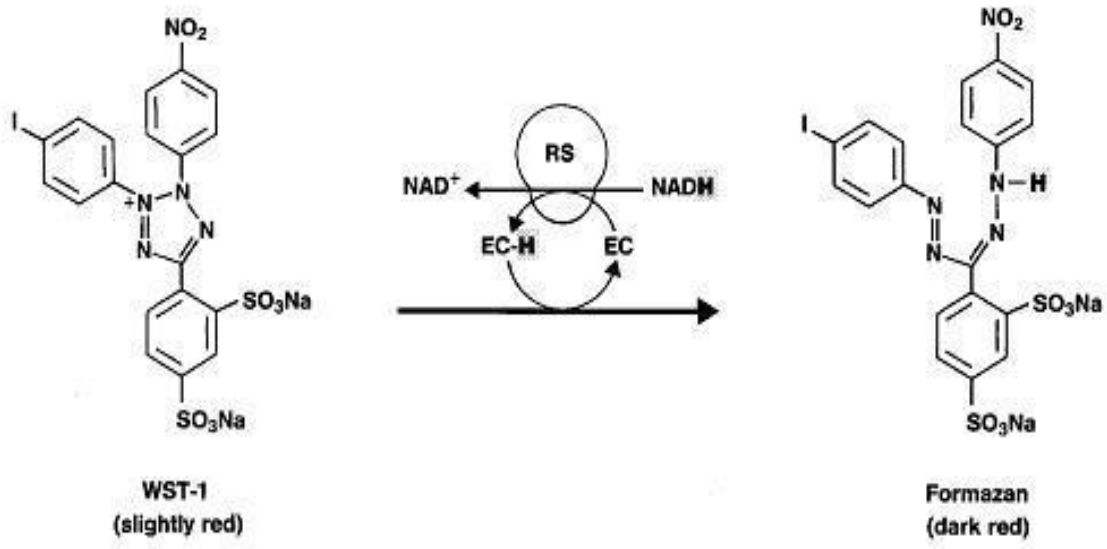
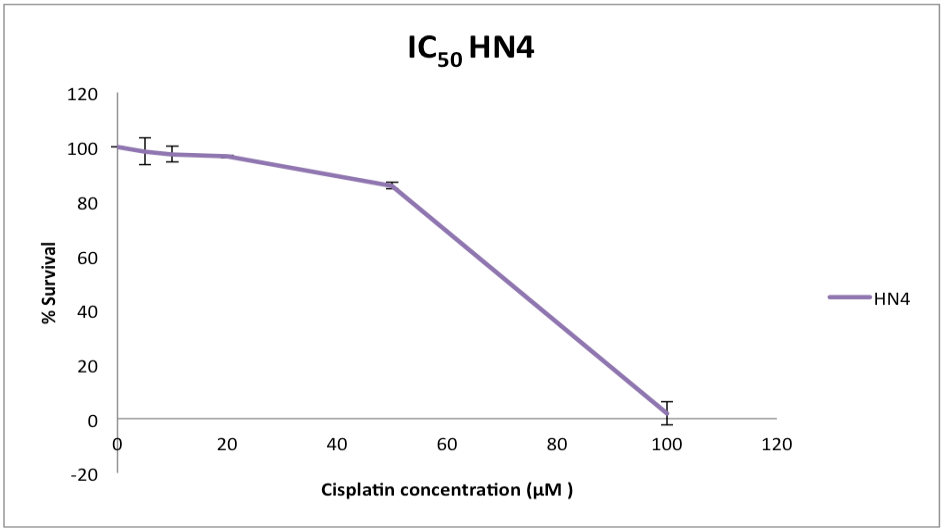
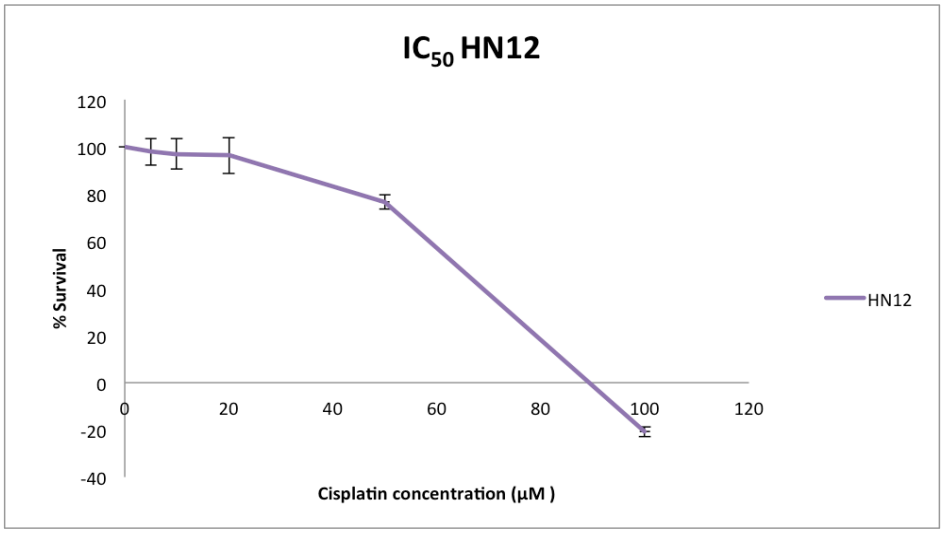


Figure 5: Cleavage of the tetrazolium salt WST-1 to formazan. (EC=electron coupling reagent, RS=mitochondrial succinate-tetrazolium-reductase system) [52]

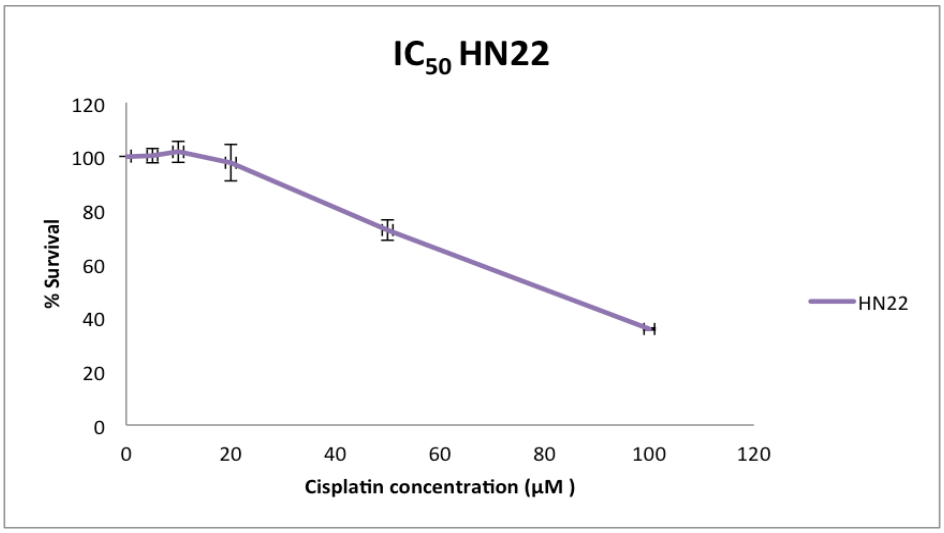
A)


IC50 HN4
120
100
80
60
40
20
0
-20
% Survival
0
20
40
60
80
100
120
Cisplatin concentration (µM)
HN4

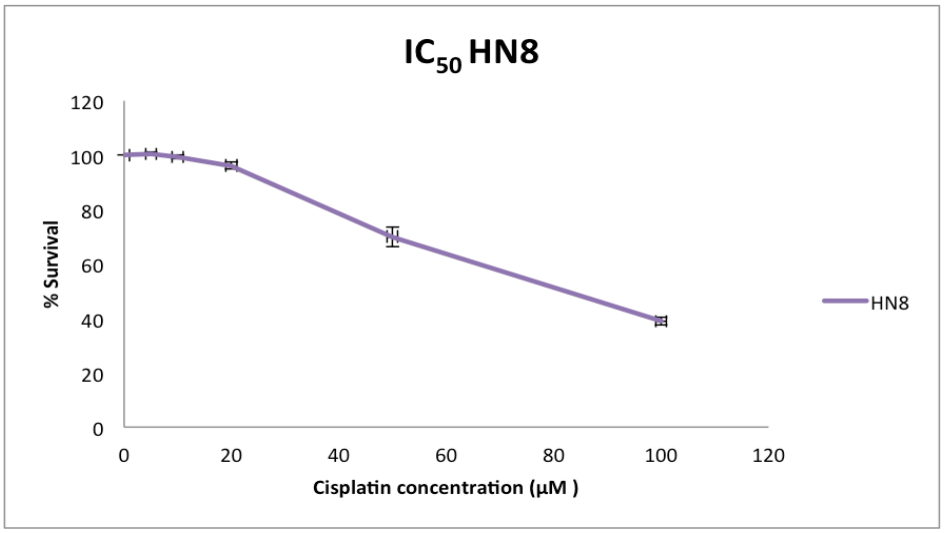
B)


IC50 HN12
120
100
80
60
40
20
0
-20
-40
% Survival
0
20
40
60
80
100
120
Cisplatin concentration (µM)
HN12

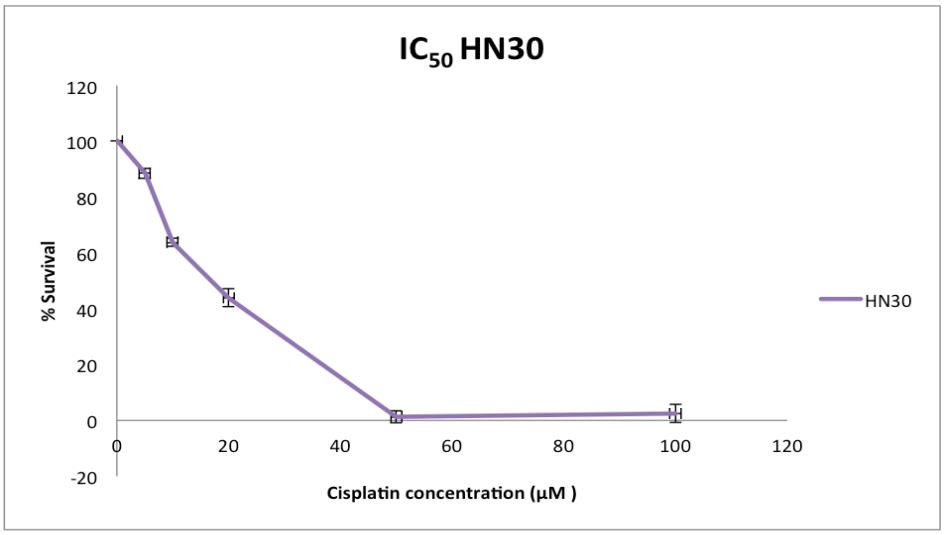
C)


IC50 HN22
120
100
80
60
40
20
0
% Survival
0
20
40
60
80
100
120
Cisplatin concentration (µM)
HN22

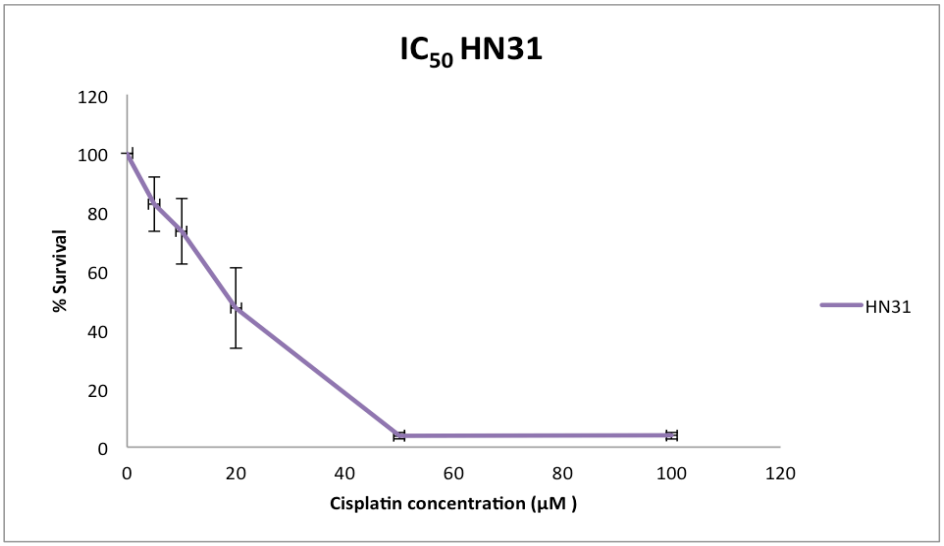
D)


IC50 HN8
120
100
80
60
40
20
0
% Survival
0
20
40
60
80
100
120
Cisplatin concentration (µM)
HN8

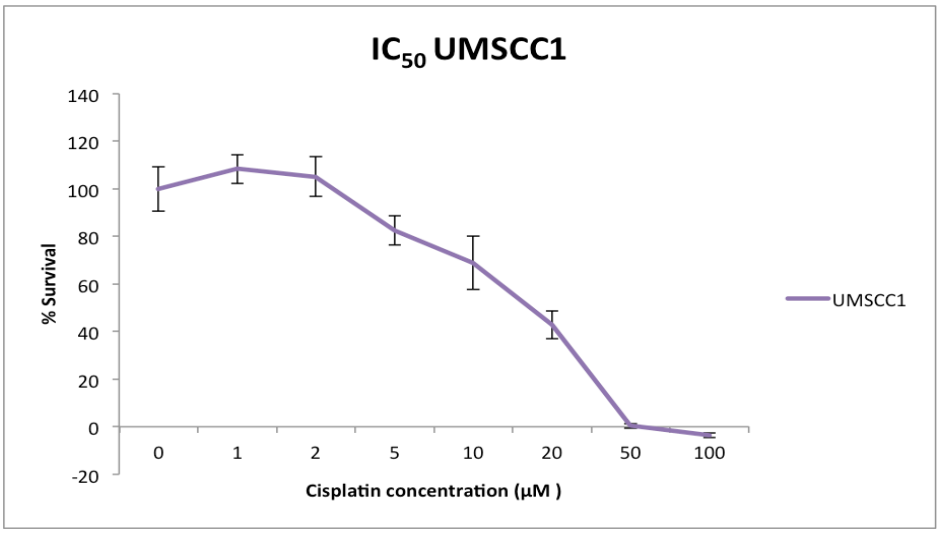
E)


IC50 HN30
120
100
80
60
40
20
0
-20
% Survival
0
20
40
60
80
100
120
Cisplatin concentration (µM)
HN30

F)


IC50 HN31
120
100
80
60
40
20
0
% Survival
0
20
40
60
80
100
120
Cisplatin concentration (µM)
HN31

G)



H)

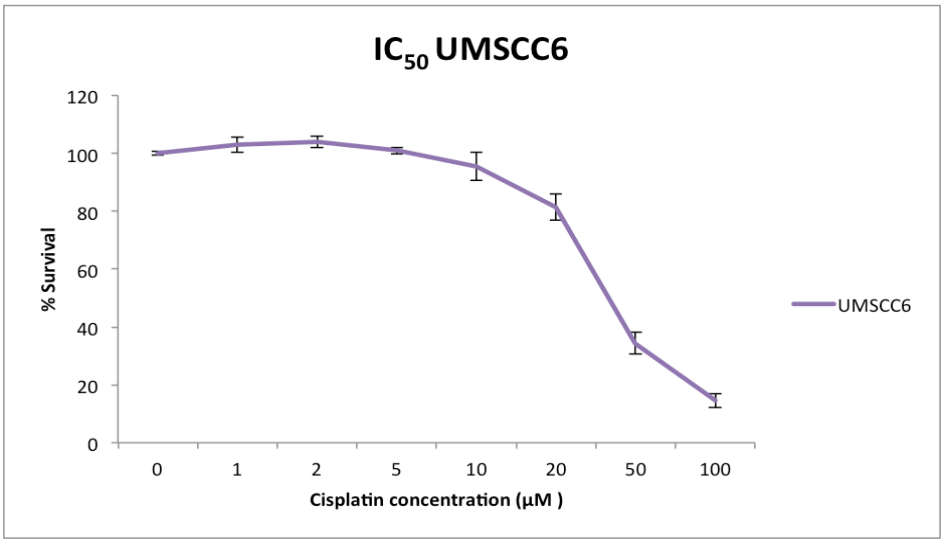


Figure 6: IC_{50} of cisplatin treatment in HNSCC cell lines. Cell proliferation was determined by the WST-1 assay. HNSCC cell lines were treated with different concentrations of cisplatin for 48 hours. The results are the mean ±S.D. of triplicates. (a) HN4 (b) HN12 (c) HN22 (d) HN8 (e) HN30 (f) HN31 (g) UMSCC1 and (h) UMSCC6 (a-f [53])

5.2 Induction of Noxa is required to induce apoptosis under cisplatin treatment in HNSCC cells

To examine whether Noxa up-regulation is required for cisplatin-induced cell death, we introduced short-hairpin RNA (shRNA) for Noxa to down-regulate Noxa expression. For a control we used a non-targeting shRNA. HN31 cells that stably expressed sh-control (sh-C) or sh-Noxa were seeded in 6-well plates and treated with cisplatin in each well (20 μM). Cells were harvested at 0, 2, 4, 8, 16, and 24 hour time points and probed with cleaved-PARP, MCL-1, and Noxa. Cleaved-PARP is one target protein of active caspase-3, which is cleaved (activated) during caspase-dependent apoptosis. Under sh-Noxa conditions there is a noticeable reduction in cleaved-PARP, indicating a reduction in apoptosis (Figure 7). This result indicates that Noxa is contributing to apoptosis as a result of cisplatin treatment in HN31 cells.

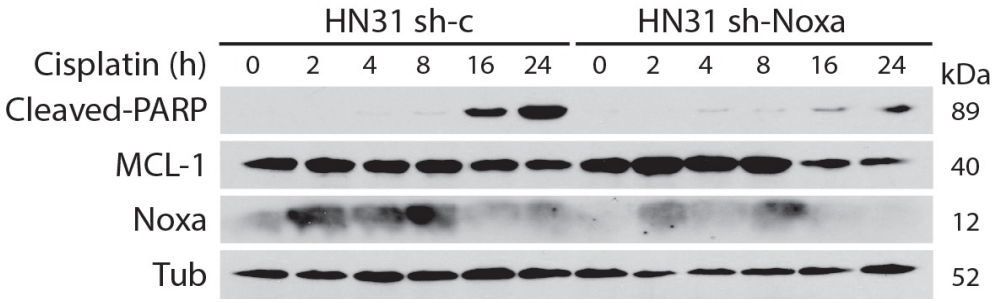


Figure 7: Cisplatin treated HN31 Noxa knockdown. HN31 cells were infected with lentivirus-encoding shRNA for non-targeting control or Noxa. The cells were then treated with cisplatin (20 μM) for the indicated periods. Equal amounts of total lysates were subjected to Western blot analysis using indicated antibodies. Alpha-tubulin was used to confirm equal loading of total lysates [53].

5.3 Noxa overexpression results in the induction of cell death in HNSCC cells

Previous research has shown that treatment of HNSCC cells with cisplatin leads to up-regulation of Noxa, which results in the sequestration and subsequent degradation of pro-survival protein MCL-1 [49]. Therefore, up-regulation of Noxa alone may be enough to induce cell death in HNSCC cells. To investigate this we infected HNSCC cell lines with lentivirus-encoding Flag-tagged Noxa and an empty vector as a control. The cells were harvested after 24 hours of incubation and Western blot analysis was utilized to examine protein expression levels. In all HNSCC cell lines tested, we observed an increase of cleaved-PARP, which is an indication that Noxa overexpression alone is capable of inducing apoptosis (Figure 8).

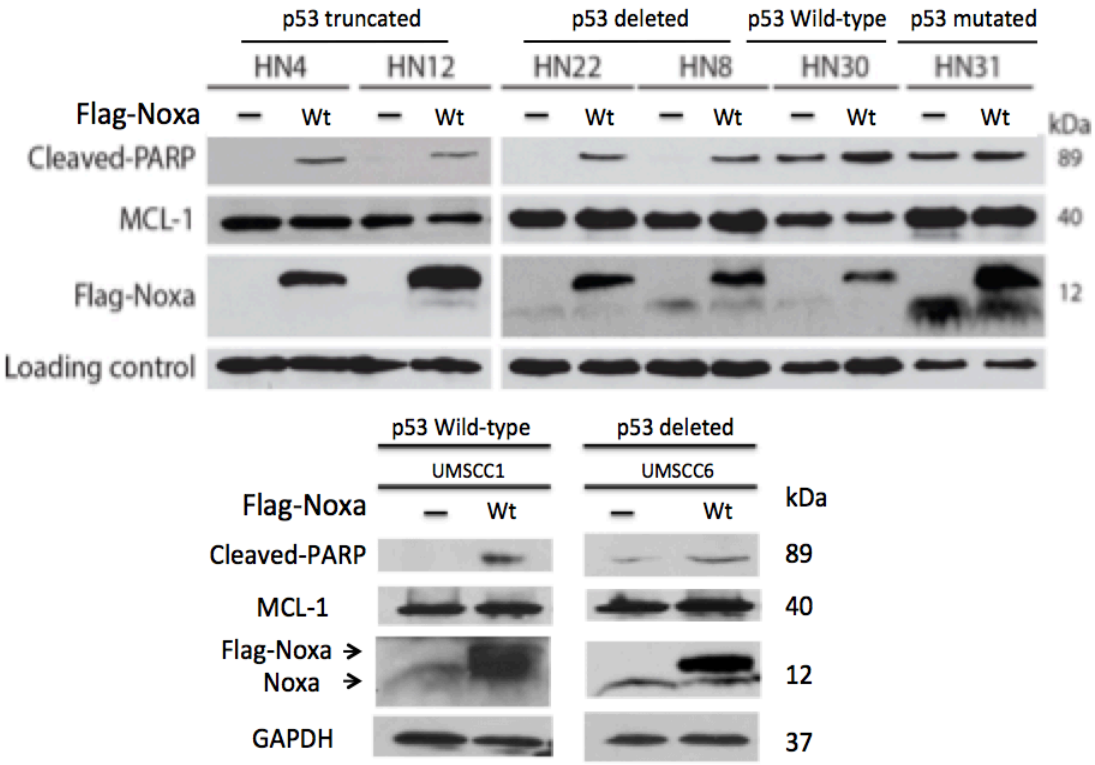


Figure 8: Overexpression of Noxa induces cell death in HNSCC cell lines. HNSCC cell lines were infected with lentivirus-encoding Flag-tagged Noxa cDNA or an empty vector as a control.

Following 24 hours of incubation the cells were harvested and equal amounts of total lysates were subjected to Western blot analysis with antibodies listed. GAPDH was used as a loading control for HN4, HN12, UMSCC1, and UMSCC6, whereas alpha-tubulin was used for HN22, HN8, HN30, and HN31. [-: control, wt: Noxa wild-type (overexpression)] (HN4, HN12, HN22, HN8, HN30, & HN31 [53])

5.4 Noxa overexpression induces apoptosis only in HNSCC cells but not in Normal Oral Keratinocytes (NOK)

Knowing that Noxa overexpression induces cell death in HNSCC cell lines, we decided to infect immortalized normal oral keratinocytes (NOK) using lentivirus-encoding Flag-tagged Noxa cDNA or an empty vector as a control. The amount of apoptosis was determined by Western blot analyses with cleaved-PARP antibody. NOK clearly exhibited less induction of cleaved-PARP compared to HN8 and HN31 cell lines (Figure 9a). Furthermore, a crystal violet staining assay was performed with HN31 cells and NOK. HN31 and NOK were infected with an empty vector as a control and Flag-tagged Noxa cDNA. Crystal violet staining assay indicated that there was a significant amount of live NOK when overexpressing Noxa compared to HN31 tumor cells (Figure 9b). These results indicate that cell death induced by Noxa overexpression is tumor-specific.

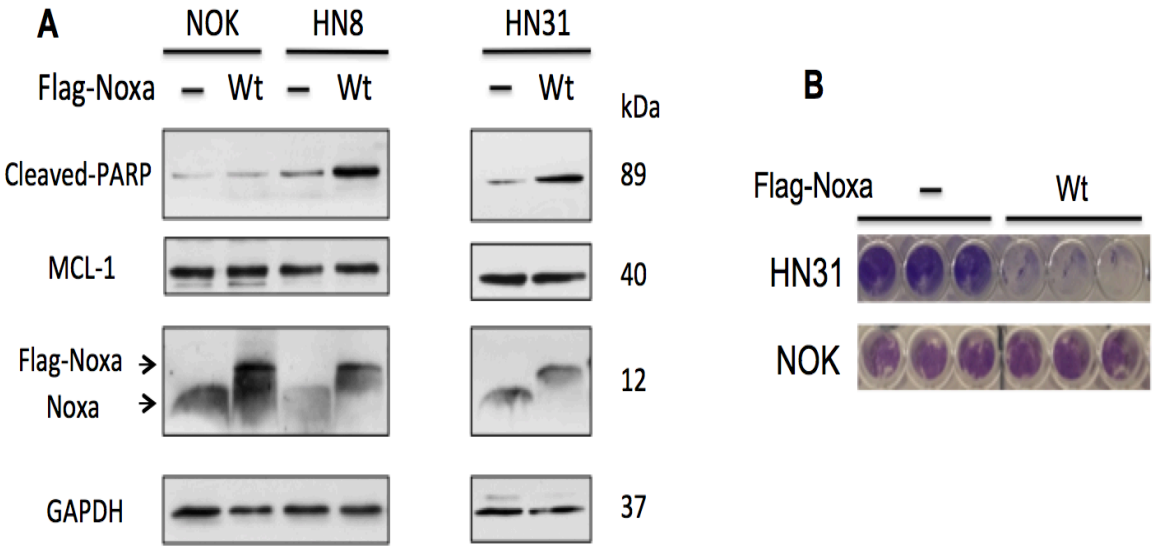


Figure 9: Noxa overexpression induces tumor-specific cell death. (**a**) NOK, HN8, and HN31 cells were infected with lentivirus-encoding Flag-tagged Noxa cDNA or an empty vector as a control. Following 24 hours of incubation, the cells were harvested and equal amounts of total lysates were subjected to Western blot analysis with antibodies listed. GAPDH was used as a loading control in all cell lines. (**b**) HN31 and NOK were seeded in a 96 well plate and infected with lentivirus-encoding Flag-tagged Noxa cDNA or an empty vector as a control. Seventy-two hours after the infection, cells were stained with crystal violet to quantitate the amount of cells still adherent to the plate (live cells). [-: control, wt: Noxa wild-type (overexpression)]

5.5 Functional BH3-domain is required for Noxa-induced cell death and binding to MCL-1 in HNSCC cells

Since Noxa is a BH3-only protein, we examined whether a functional BH3-domain is required for Noxa-induction of cell death. Therefore, we created three amino acid mutations within the BH3-domain of Noxa (3E mutant). Hydrophobic leucines and phenylalanine in the BH3 domain were replaced with hydrophilic glutamic acids to prevent α-helical structure (Figure 10b). HN31 and HN8 cell lines were infected with lentivirus-encoding 3E cDNA, Noxa cDNA as positive control, or an empty vector as negative control. Western blot analysis using cleaved-PARP antibody was implemented to determine the amount of cell death. The 3E mutant infection to HN31 and HN8 cell lines revealed significantly less cleaved-PARP than those with Noxa wild-type (Figure 10a). This data suggests that a functional BH3-domain of Noxa is required to induce cell death through binding of MCL-1 in HNSCC cell lines. Next, we wanted to confirm that the reason we examined no cell death, was due to the Noxa mutated BH3 domain being unable to sequester and bind to MCL-1 and inhibit its function. To analyze this we infected HN31 cells with lentivirus-encoding Noxa wt cDNA or 3E cDNA and examined the protein-protein interactions with immunoprecipitation followed by Western blot analysis. We observed a binding of MCL-1 and Noxa in the wild-type Noxa infection, but no binding of the 3E mutated Noxa infection (Figure 10c), suggesting that Noxa needs a functional BH3 domain to induce cell death through the binding to MCL-1.

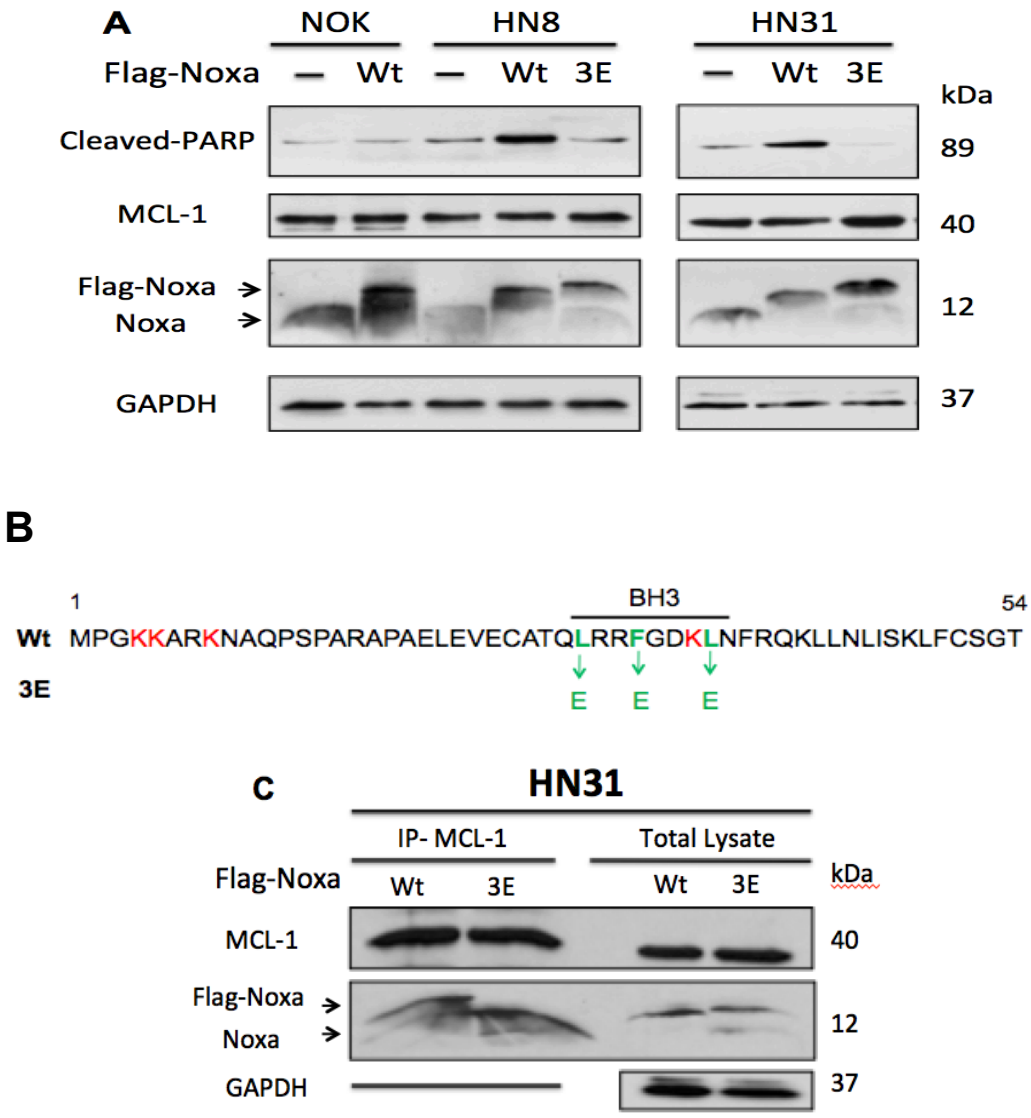


Figure 10: Noxa 3E mutant results in no induction of cell death. (**a**) HN8 and HN31 cells were infected with lentivirus-encoding a Noxa BH3-domain mutant (3E). Infected cells were harvested after 24 hours of incubation and equal amounts of total lysates were subjected to Western blot analysis with antibodies indicated. GAPDH was used as a loading control. [-: control, wt: Noxa wild-type (overexpression), 3E: Noxa protein with a mutated BH3 domain] (**b**) The amino acid sequence of human Noxa protein. The green arrows point to the amino acids that were replaced in the BH3-domain of Noxa. (**c**) HN31 cells were infected with lentivirus-

encoding a Flag-tagged Noxa wt cDNA and Noxa BH3-domain mutant (3E). Infected cells were harvested following 24 hours of incubation and an immunoprecipitation was conducted, followed by Western blot analysis. GAPDH was utilized as a loading control.

5.6 Noxa overexpression results in reduction of MCL-1/BAK interaction and activation of BAK and BAX

Knowing that pro-survival MCL-1 and BCL-X_L normally bind to and inhibit the pro-apoptotic function of BAK, we investigated whether there would be a change in protein interactions upon Noxa overexpression. To do this we overexpressed Noxa in HN31 cells by infecting them with lentivirus-encoding Flag-tagged Noxa cDNA or an empty vector as a control. To analyze the protein-protein interactions we conducted an immunoprecipitation followed by Western blot analysis. We observed a decrease in MCL-1 and BAK interaction when Noxa was overexpressed, suggesting that Noxa is causing the release of BAK upon its binding to MCL-1 (Figure 11a). At the same time we saw that BCL-X_L and BAK interaction is unchanged by Noxa overexpression, indicating that Noxa is primarily acting through its sequestration of MCL-1.

In healthy cells BAX and BAK adopt globular α-helical structures as monomers, which hide their N-terminus portion of the protein. Following a variety of stress signals such as cisplatin treatment or Noxa overexpression, they undergo a conformational change (activation), allowing them to become pore-forming proteins of the outer mitochondrial membrane. During this conformational change of BAX and BAK, their N-terminus becomes exposed. To confirm that BAK is activated when released from MCL-1, we used a monoclonal N-terminus specific antibody for immunoprecipitation and then analyzed the change of protein activation with a

Western blot (Figure 11b). When overexpressing Noxa, BAK underwent strong activation. We also analyzed BAX activation under Noxa overexpression by using an N-terminus specific antibody for immunoprecipitation followed by Western blot analysis (Figure 11c). The result indicated a substantial increase in BAX activation, suggesting that Noxa is able to influence the amount of activated BAX. We then examined the possibility whether Noxa overexpression changed the interaction of BAX and MCL-1 in HN31 cells. We confirmed this by performing immunoprecipitation followed by Western blot analysis. We observed that the interaction between MCL-1 and BAX was undetectable in HN31 cells either with an empty vector or with Noxa expression (Figure 11d).

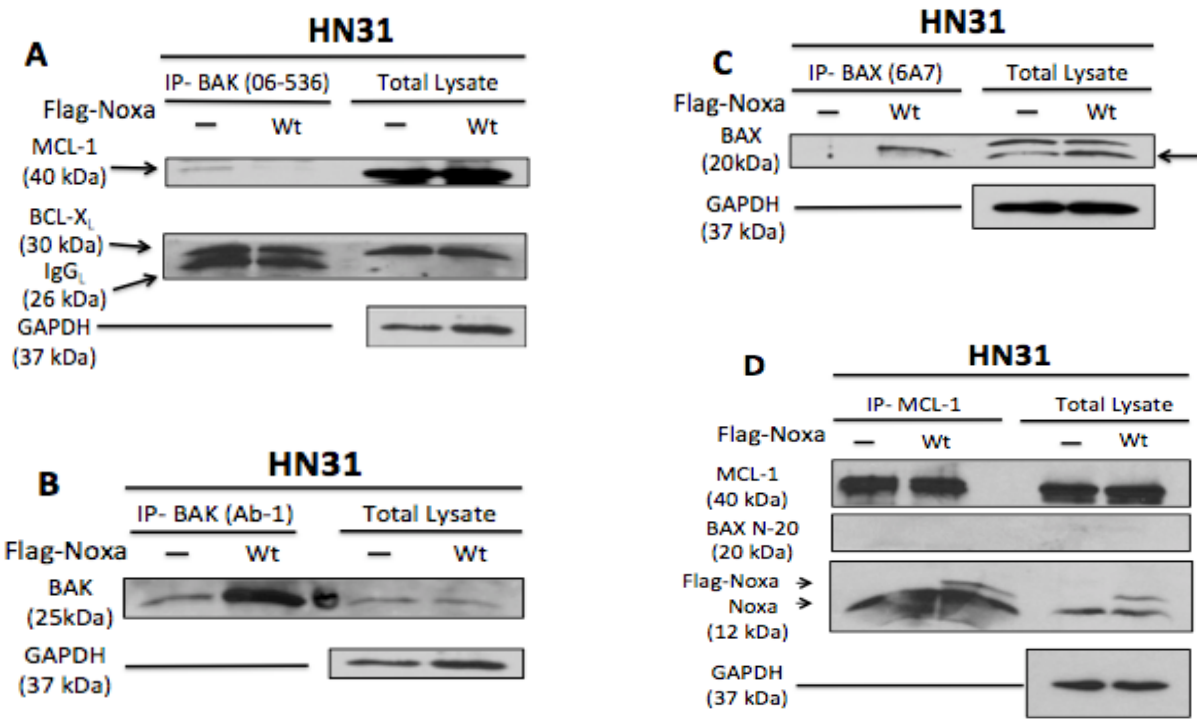


Figure 11: Immunoprecipitations to analyze BCL-2 family interactions and activation following Noxa overexpression. HN31 cells were infected with lentivirus-encoding Flag-tagged Noxa cDNA or an empty vector as a control. The infected cells were harvested after 24 hours of incubation. GAPDH was used for all Western blots to confirm equal amounts of total lysate loaded for the experiment. (**a**) HN31 infected cells were harvested and immunoprecipitation was performed with a BAK antibody followed by Western blot analysis with indicated antibodies. (**b**) HN31 infected cells were harvested to analyze BAK activation. Total cell extracts were subjected to immunoprecipitation with BAK (conformational specific) antibody followed by Western blot analysis with the indicated antibodies. (**c**) HN31 infected cells were harvested to analyze BAX activation. The harvested cells were used for immunoprecipitation with a BAX (conformational specific) antibody followed by Western blot analysis with indicated antibodies. (**d**) HN31 infected cells were harvested and immunoprecipitation was performed with an MCL-1 antibody. Immunoprecipitated extracts and total lysates were then loaded into a Western blot to

examine the interaction of MCL-1 and BAX. [-: control, wt: Noxa wild-type (overexpression)]

5.7 Down-regulation of BAX and BAK inhibits cell death induced by Noxa overexpression

With a clear increase in BAK and BAX activation by Noxa overexpression in HNSCC cells, we then investigated the contribution of each protein to Noxa-induced cell death. To examine this we infected HN31 cells with lentivirus-encoding short-hairpin RNA (shRNA) for non-targeting sh-control (shC), BAX, or BAK to establish cells with stable knockdowns. The HN31 shC, shBAX, and shBAK cells were seeded in a 6 well plate and infected with lentivirus-encoding Flag-tagged Noxa cDNA or an empty vector as a control. Cells were harvested and cell death was analyzed with Western blot analyses (Figure 12a). Both HN31 shBAX and shBAK cells showed a reduction of cleaved-PARP compared to HN31 shC cells. This data suggests that both BAX and BAK play roles in Noxa-mediated cell death. Moreover, we conducted a double-knockdown experiment in which both BAX and BAK were down-regulated. The stable HN31 shBAX cells were seeded in a 6 well plate and then infected lentivirus with shBAK to achieve a transient knockdown while using shC as a control. Twenty-four hours later, the cells were infected with lentivirus-encoding Flag-tagged Noxa cDNA or empty vector as control. Another 24 hours later, the cells were harvested and equal amounts of total lysates were subjected to Western blot analysis (Figure 12b). The double-knockdown of BAX and BAK in HN31 cells resulted in no induction of cleaved-caspase-3, which is an indicator of apoptosis. This result suggests that Noxa is primarily inducing cell death through both BAX and BAK activation in HNSCC cells.

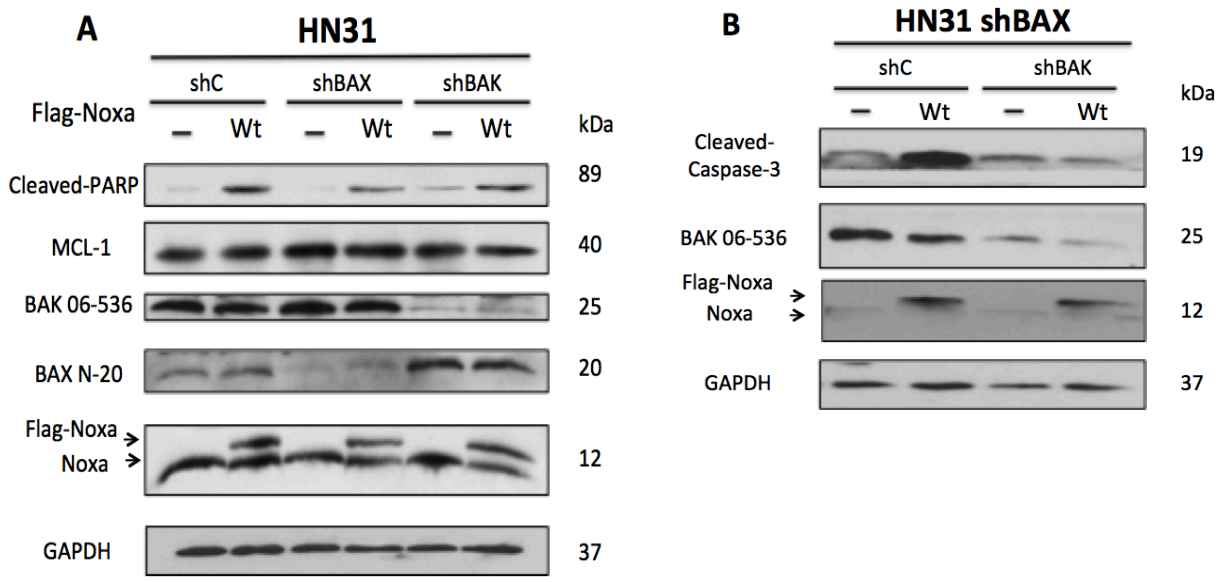


Figure 12: Knockdown of BAX and BAK reduces Noxa-induced cell death. (**a**) HN31 cells were infected with lentivirus-encoding short-hairpin RNA (shRNA) for non-targeting sh-control (shC), BAX, or BAK to establish stable knockdown cells. Cells were then infected with lentivirus-encoding Flag-tagged Noxa cDNA and an empty vector as a control. Equal amounts of total lysates were subjected to Western blot analysis with the indicated antibodies. (**b**) HN31 shBAX cells were transiently infected with lentivirus-encoding shRNA for non-targeting control or BAK. Cells were then infected with lentivirus-encoding Flag-tagged Noxa cDNA or an empty vector as a control. Equal amounts of total lysates were subjected to Western blot analysis using indicated antibodies. GAPDH was used as a loading control for both Western blots. [-: control, wt: Noxa wild-type (overexpression)]

5.8 Noxa expression not induced in cisplatin-resistant isogenic cell lines

Knowing that prolonged exposure to cisplatin often results in acquired resistance in tumor, we set out to establish cisplatin resistant cell lines so that we may analyze the mechanisms that led to resistance. We treated HNSCC cell lines with low doses of cisplatin for a prolonged period of time, and monitored their increase in resistance by performing WST-1 assays after treatment. We were able to establish a isogenic cisplatin-resistant cell line from HN31 cells. HN31 and HN31R cell lines were seeded in 6 well plates and treated with their IC_{50} cisplatin concentrations (20 μM for HN31). Cells were harvested at 0, 8, 16, and 24 hour after the treatment and total lysates were equally loaded into Western blots to examine the differences in protein expressions between the parental and resistant cell line (Figure 13a). In HN31R cells, we observed a reduction of cleaved-PARP compared to the parental cell line (HN31). This data indicates that the acquired resistant cells are undergoing less apoptosis than their isogenic parental counterparts. Furthermore, the resistant cells both showed less induction of pro-apoptotic protein Noxa, which may be attributing to this decrease in apoptosis within those cells. Observing this, we decided to investigate whether Noxa overexpression in these resistant cells would be capable of inducing cell death. To examine this we infected the HN31R cell line with lentivirus-encoding Flag-tagged Noxa cDNA or an empty vector as a control. Equal amounts of the total lysates were subjected to Western blot analysis to detect the changes in protein expression levels between the HN31 parental cell and the HN31R cell (Figure 13b). Following Noxa overexpression we observed no significant induction of cleaved-PARP to indicate increased cell death. We also examined the expression levels of other BCL-2 family proteins and noticed that nearly all of them were either more or less expressed when compared to the parental cell line.

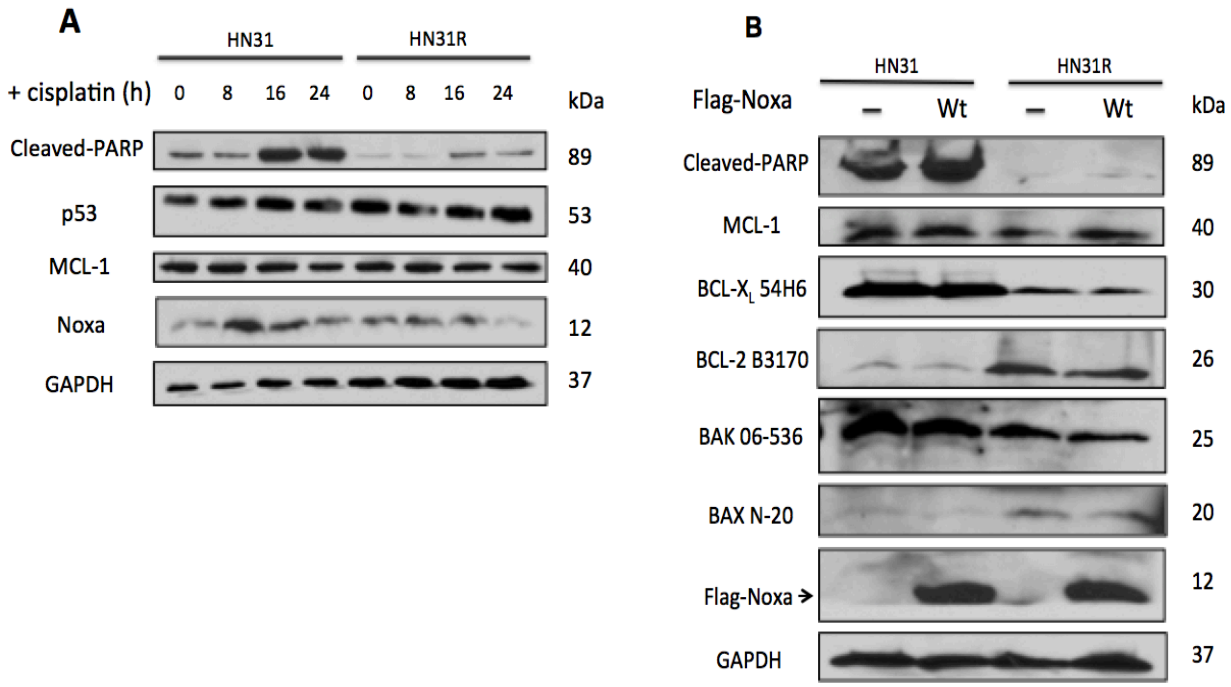


Figure 13: HN31R resistant cell line shows little response to cisplatin or Noxa overexpression. (**a**) HN31 and HN31R cells were seeded and treated with 20 μM of cisplatin and then harvested following 0, 8, 16, and 24 hours of exposure. Equal amounts of the total lysates were subjected to Western blot analysis with the indicated antibodies. (**b**) HN31 and HN31R cells were infected with lentivirus-encoding Flag-tagged Noxa cDNA or an empty vector as a control. The infected cells were harvested after 24 hours of incubation. Equal amounts of the total lysates were subjected to Western blot analysis with the indicated antibodies. GAPDH was used as a loading control for both Western blots.

DISCUSSION

Cisplatin treatment of HNSCC results in DNA damage, which then induces p53 to prevent further DNA replication or induce apoptosis if DNA is unable to be prepared. When determining IC_{50} of the HNSCC cell lines tested, we observed that the IC_{50} of p53 wild-type cell lines (HN30 and UMSCC1) tended to possess a higher sensitivity to cisplatin than that of p53 deleted and non-functional p53 cell lines (Table 1 and Figure 6). This data suggest that activation of the p53-dependent pathway could sensitize the cells to cisplatin treatment compared in the p53 deleted or non-functional cell lines.

The BCL-2 family members are the primary guardians of mitochondria-dependent apoptosis. Cisplatin, a chemotherapeutic drug, has been shown to induce cell death through mechanisms other than apoptosis [45,46]; however, our results indicate that the cisplatin-triggered cell death is mainly through BCL-2 family-dependent mitochondrial apoptosis. Of these BCL-2 family proteins, Noxa is up-regulated during cisplatin treatment in HNSCC cell lines. When treating HN31 shNoxa cells with cisplatin, we observed a decrease in cleaved-PARP when compared to the control cells (Figure 7). This data confirms that Noxa is important for cisplatin-induced cell death in HNSCC cell lines. Similar to HNSCC, Noxa has also been shown to be up-regulated during cisplatin-induced cell death in breast and cervical cancer cell lines [44]. Perhaps there are even more types of cancer in which Noxa plays a critical role chemotherapeutic-induced cell death.

Our data showed that Noxa overexpression alone is capable of inducing cell death in HNSCC cell lines (Figure 8). With Noxa overexpression, the level of cleaved-PARP was increased in each cell line regardless of p53 status. Furthermore, we determined that Noxa-induced cell death is tumor specific by analyzing the immortalized normal oral keratinocytes

(NOK). When overexpressing Noxa in NOK, we detected no induction of cleaved-PARP compared to that in the control NOK (Figure 9). While the mechanism is not yet known as to why Noxa-induced cell death is tumor specific, this data contributes to a potential of using Noxa as a therapeutic strategy to treat head and neck cancer. Future research with Noxa overexpression in other non-transformed cell lines would certainly provide greater support to our current results.

We then examined whether the BH3 domain of Noxa is required to induce cell death in HNSCC cells. When transfecting the Noxa 3E mutant Noxa into HN8 and HN31 cells, we observed no induction of cleaved-PARP with the 3E mutant compared to the control. These results indicate that the BH3 domain of Noxa is essential for Noxa-induced cell death in HNSCC cells (Figure 10a). Furthermore, we confirmed that the 3E Noxa mutant is unable to bind to MCL-1 to allow the release of BAK and subsequent apoptosis (Figure 10c). Others have also shown that few mutations within the BH3 domain of BCL-2 proteins will alter the selectiveness of the protein and their ability to induce apoptosis [48]. With this information, we are developing a peptide of Noxa BH3 domain to maintain Noxa's function in inducing apoptosis. This BH3 peptide would minimize side effects on tumor surrounding tissues by only inhibiting the BCL-2 pro-survival protein MCL-1. Using the peptide when treating head and neck cancers that rely on their development by MCL-1 up-regulation could prove useful. For example, MCL-1 up-regulation has been identified in in 9% of luminal B breast cancers [56] and 54% of triple negative breast cancers after treatment with neoadjuvant chemotherapies [57].

Under survival conditions it is known that MCL-1 and BCL-X_L are bound to BAK to inhibit apoptosis. These interactions can occur with the BAK BH3 domain, which is also required for the BAK oligomerization and induction of mitochondrial-dependent apoptosis [49]. Following up-regulation of Noxa, MCL-1 is sequestered to the mitochondria and targeted for

degradation, allowing BAK to be displaced to induce apoptosis. When Noxa was overexpressed, the interaction of BAK and MCL-1 decreased, which indicates that up-regulation of Noxa results in the release of BAK from MCL-1 to induce cell death (Figure 11a). Furthermore, by using a conformational specific BAK antibody we were able to confirm that BAK was activated by Noxa overexpression, which is critical for BAK dimerization and outer mitochondrial pore formation (Figure 11b). Interestingly, we also observed BAX activation when Noxa was overexpressed (Figure 11c). With this information we then confirmed that there was no interaction between BAX and MCL-1 (Figure 11d). Knowing that MCL-1 and BAX do not interact, we can conclude that Noxa is functioning through some independent mechanism of MCL-1 sequestration to activate BAX. Still, the mechanism as to how Noxa induces BAX activation is not yet known. Other BH3-only proteins may sequester BCL-X_L or BCL-2 to induce BAX activation, since Noxa is not capable of providing that function [47,48]. Thus, immunoprecipitation with other BCL-2 family members and their interaction with BAX with and without Noxa overexpression may provide explanation for this phenomenon.

Knowing that BAX and BAK are activated upon Noxa overexpression, we determined whether Noxa is primarily dependent on their activation to induce cell death. When performing single knockdowns of BAX and BAK, the levels of cleaved-PARP were reduced partially in both cells compared to those in control cells (Figure 12a). Furthermore, we performed double knockdowns of BAX and BAK (Figure 12b). Under these conditions we observed no induction of cleaved-caspase-3, which suggest that Noxa is primarily inducing cell death through the BAX and BAK-dependent pathway. Future research should confirm this data by performing Annexin V and propidium iodide staining followed by flow cytometry (FACS analysis) to quantify this reduction in cell death observed in our Western blot analyses.

Over a prolonged exposure to chemotherapeutic agents, patients often experience tumor-acquired resistance. Therefore, we attempted to mimic this situation *in vitro* by treating HNSCC cells with low doses of cisplatin for several weeks. When treating the resistant cells (HN31R) with cisplatin, our results show a decrease in cleaved-PARP compared to the parental HN31 cell line (Figure 13a). Furthermore, endogenous Noxa expression levels were decreased in HN31R cells. When investigating whether Noxa overexpression was capable of recovering cell death in HN31R cells, we observed no increase of cleaved-PARP in Noxa overexpressed cells compared to that in control cells (Figure 13b). When analyzing the expression of other BCL-2 family proteins, we noticed that the expression levels in HN31R cells were altered compared to those in the parental HN31 cells. Among the pro-survival proteins, the levels of BCL-X_L and MCL-1 appear reduced, but the level of BCL-2 is greatly increased in HN31R cells. Pro-apoptotic BAK exhibits a reduced expression, but BAX expression is increased in the resistant cells. Upon these results, it is currently unclear as to what change in machinery is playing a role in acquired resistance within this HNSCC cell line. Further research is needed to elucidate the genetic mutations in the HN31R cell line. Combination therapies with BCL-2 pro-survival protein inhibitors should also be investigated whether there is a recovery of cell death in the resistant cell line. In particular, a combination of Noxa overexpression and ABT-263 (BCL-2 and BCL-X_L inhibitor) may be sufficient to induce cell death in HN31R cells.

In summary, our results indicate five important findings. First, Noxa induction is required for cisplatin-induced cell death in HNSCC cell lines. Second, Noxa overexpression alone is capable of inducing tumor-specific cell death in HNSCC cell lines. Third, the BH3 domain of Noxa is required for cell death induction through the inhibition of MCL-1 function. Fourth, Noxa-mediated cell death is primarily activated through BAX and BAK. Fifth, the cisplatin-

resistant isogenic HN31 cell line is not as sensitive to Noxa overexpression as its parental cell line. These insights may be useful for the development of novel therapeutic strategy to treat head and neck cancer.

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